Yeast mitochondrial DNA mutators with deficient proofreading exonuclease activity

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The MIPI gene which encodes yeast mitochondrial DNA polymerase possesses in its N-terminal region the three motifs (Exo1, Exo2 and Exa3) which characterize the 3′→5′ exonucleyotic domain of many DNA polymerases. By site-directed mutagenesis we have substituted alanine or glycine residues for conserved aspartate residues in each consensus sequence. Yeast mutants were therefore generated that are capable of replicating mitochondrial DNA (mtDNA) and exhibit a mutator phenotype, as estimated by the several hundred-fold increase in the frequency of spontaneous mitochondrial erythromycin resistant mutants. By overexpressing the mtDNA polymerase from the GAL1 promoter as a major 140 kDa polypeptide, we showed that the wild-type enzyme possesses a mismatch-specific 3′→5′ exonuclease activity. This activity was decreased by ~500-fold in the mutant D347A; in contrast, the extent of DNA synthesis was only slightly decreased. The wild-type mtDNA polymerase efficiently catalyses elongation of singly-primed M13 DNA to the full-length product. However, the mutant preferentially accumulates low molecular weight products. These data were extended to the two other mutants D171G and D230A. Glycine substitution for the Cys344 residue which is present in the Exo3 site of several polymerases generates a mutant with a slightly higher mtDNA mutation rate and a slightly lower 3′→5′ exonucleyotic activity. We conclude that proofreading is an important determinant of accuracy in the replication of yeast mtDNA.

Key words: mutators/mtDNA polymerase/proofreading/processivity

Introduction

The polypeptides encoded by mitochondrial DNA (mtDNA) are small in number but essential to respiration and oxidative phosphorylation. Therefore the integrity of mitochondrial genetic information must be maintained. There are two main mechanisms by which DNA polymerases ensure accurate DNA replication: high selectivity of nucleotides during the polymerization reaction and proofreading. Animal mtDNA polymerases have a physically associated 3′→5′ exonuclease proofreading activity (Kunkel and Soni, 1988; Insdorf and Bogenhagen, 1989; Kaguni and Olson, 1989; Kunkel and Mosbaugh, 1989). It is unknown, however, whether this activity belongs to the polymerase subunit and what role it plays in vivo.

We have previously cloned and sequenced the nuclear MIPI gene encoding the catalytic subunit of yeast mitochondrial DNA polymerase (Foury, 1989). Although it is highly divergent from other known eukaryotic and prokaryotic DNA polymerases, the yeast mtDNA polymerase seems to share a common ancestor with prokaryotic DNA polymerases (Ito and Braithwaite, 1989; Blanco et al., 1991; Ito and Braithwaite, 1991) and is also related to the DNA polymerases of the Kluyveromyces lactis killer factors (Tommasino et al., 1988; Foury, unpublished results). Moreover, we and others (Foury and Vanderstraeten, 1990; Ito and Braithwaite, 1990; Blanco et al., 1991) have found that the three consensus sequences which characterize the 3′→5′ exonucleyotic domain of many prokaryotic and eukaryotic DNA polymerases (Bernad et al., 1989) are present in the N-terminal part of the MIPI gene. In this work, we have addressed the following questions: does yeast mtDNA polymerase possess a 3′→5′ exonucleyotic domain and, if so, does 3′→5′ exonuclease activity play an important role in the correction of errors produced during mtDNA replication in vivo?

Results

Overproduction of the mtDNA polymerase from the GAL1 promoter

We have previously reported that the gap filling activity of the mtDNA polymerase was 30- to 40-fold higher in yeast transformants carrying the MIPI gene on a multicopy plasmid than in a wild-type strain harbouring a single copy of the MIPI gene (Foury, 1989). Therefore, in order to increase further the mtDNA polymerase activity in soluble mitochondrial extracts, we decided to overproduce the MIPI product from the strong inducible GAL1 promoter on a multicopy plasmid. The MIPI gene was fused to the GAL1 promoter, 45 bp upstream of the MIPI translation initiation codon, as described in Materials and methods (Figure 1A). Because yeast mitochondria possess a potent endo-exonuclease activity (encoded by the NUC1 gene) (Zassenhaus et al., 1988) which might interfere with the mtDNA polymerase assays, transformation experiments were achieved in the strain A25 that carries a null nuc1 allele. After a 16 h induction in galactose minimal medium, soluble mitochondrial extracts were prepared in the presence of a cocktail of protease inhibitors. Electrophoresis in SDS-polyacrylamide gels revealed the presence of major polypeptide at ~140 kDa (the expected size), while no polypeptide was detected with the insertless pLGALZ3 vector used as a negative control (Figure 1B, panel a). Thus, the MIPI gene is translated as a single 140 kDa polypeptide.

3′→5′ exonuclease activity on a mismatched substrate

The 3′→5′ exonuclease activity on a mismatched 3′-hydroxyl terminus was measured by using two different primer
template substrates in parallel. The first consisted of a perfectly complementary 17mer oligonucleotide annealed with an M13mp19 ssDNA template; the second substrate was identical except that the primer had a single mismatch (dAMP.dAMP) at its 3'-hydroxyl terminus. In a strain harbouring a single copy of the MIP1 gene, the 3'-5' exonuclease activity was under the limits of detection (data not shown). In extracts of the strain A25 which was over-expressing the mtDNA polymerase from the GAL1 promoter, excision of both correctly paired and mispaired nucleotides at the 3'-hydroxyl terminus occurred (Figure 2A). After an incubation for 1 min in the presence of 0.01 units of enzyme 5% of the correctly paired primer and 15% of the mispaired one were converted to 16mer or lower molecular weight products (Figure 2B). Thus, the mispaired nucleotide was excised more rapidly than the correctly paired nucleotide, as expected for an editing function of the 3'-5' exonuclease. These results show that yeast mitochondrial DNA polymerase has an associated 3'-5' exonuclease activity that preferentially removes mismatched nucleotides. Products of 13-14 nucleotides in length accumulated at four consecutive cytosine/guanosine bases. This indicates that although frayed DNA is not necessarily required, the exonucleolytic activity might be impeded at GC-rich regions which have stronger hydrogen bondings.

**Isolation of mip1 mutants with a mutator phenotype**

It has been shown that the sequences of prokaryotic and eukaryotic DNA polymerases exhibiting 3'-5' exonuclease activity share three motifs with the 3'-5' exonucleolytic
domain of *Escherichia coli* DNA polymerase I (PolI) (Bernad et al., 1989). These motifs, named Exo1, Exo2 and Exo3, are located in the N-terminal region of the Klenow fragment and mainly characterized by conserved tyrosine/phenylalanine residues or glutamate/aspartate residues. The acidic residues, whose carboxylate groups are involved in dNMP and divalent metal ion binding, play an essential role in the catalytic activity of the 3'-5' exonuclease (Ollis et al., 1985; Joyce and Steitz, 1987; Derbyshire et al., 1988; Freemont et al., 1988; Derbyshire et al., 1991).

These three motifs are clearly present in the *MIP1* gene within a region spanning amino acid residues 170—347

![Fig. 2. Mismatch-specific 3'-5' exonuclease activity of wild-type and D347A mutant polymerases overexpressed from *GAL1* promoter.](image)

Extracts were prepared from strain A25 with pLGALMIP1 or pLGALD347A plasmids as in Figure 1. The reactions were performed as described in Materials and methods with 4 µM 13mp19 ssDNA substrate annealed with paired (P, 280 c.p.m./pmol) or mispaired (M, 210 c.p.m./pmol) oligonucleotides. Aliquots of 5 µl (~3000 c.p.m.) were subjected to electrophoresis. Polymerase activities in the 50 µl assays were as follows: wild-type (0.01 units), D347A (0.02 units). A. Autoradiography of the gel. It must be noted that the controls at time zero are slightly contaminated with 16mer and 18mer products. B. Quantification of the disappearance of the 17mer substrate. The data are expressed as the percentage of digested 17mer oligonucleotide per 0.01 units of polymerase. After 16 min, only 0.5% of the 17mer had been digested in the mutant. Quantification of the data was carried out by elution of the silver grains from the film as reported in Materials and methods.

Fig. 3. Site directed mutagenesis of conserved residues in the 3'-5' exonuclease consensus sequences. The nucleotide sequence is numbered according to Foury (1989). The previously published sequence had to be corrected: nucleotides 1576 and 1577 are G and T, respectively. Nucleotide substitution is indicated above the sequence and amino acid substitution is indicated below the sequence. The names and alleles of the mutants that were generated are detailed in Table III in Materials and methods.

**Table I.** Frequency of mitochondrial DNA mutations in wild-type strain and mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number of E6 colonies per 10^8 cells</th>
<th>%rho-</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIP1</td>
<td>0.3 ± 0.1 (4)</td>
<td>0.5</td>
</tr>
<tr>
<td>D171G</td>
<td>63 ± 18 (3)</td>
<td>4.1</td>
</tr>
<tr>
<td>D230A</td>
<td>77 ± 12 (3)</td>
<td>5.6</td>
</tr>
<tr>
<td>D347A</td>
<td>39 ± 13 (3)</td>
<td>7.9</td>
</tr>
<tr>
<td>C344G</td>
<td>3.5 ± 0.7 (2)</td>
<td>0.6</td>
</tr>
</tbody>
</table>

The experiments, carried out with the rho+ strain W303-1B/Lampa1-01 harbouring MIP1/PFL39 (wild-type) or mip1/PFL39 (mutant) plasmids, were performed as described in Materials and methods. Mean and standard deviation of E6 mutation frequency are given. Numbers in parentheses indicate the number of independent experiments. The percentage of rho- mutants was estimated from scoring between 1000 and 2000 colonies.

(Foury and Vanderstraeten, 1990; Ito and Braithwaite, 1990; Blanco et al., 1991). In order to determine whether they are really involved in the 3'-5' exonuclease activity, site directed mutagenesis of conserved residues was carried out (Figure 3). Alanine substitution was generated at Asp230 and Asp347 residues (Exo2 and Exo3, respectively) and a glycine residue was substituted for Asp171 and Cys344 residues (Exo1 and Exo3, respectively). The cysteine residue is conserved among viral DNA polymerases (see Bernad et al., 1989) and is present in the DNA polymerase of *K. lactis* killer factors. It is absent, however, from prokaryotic DNA polymerases. After mutagenesis, each mutant mip1 allele was introduced into a centromeric vector and the resulting plasmids were used to transform a null mip1 strain. Since a null mip1 strain has no mtDNA (rho0), transformants remained devoid of mtDNA whether their mtDNA polymerase was functional or not. It was therefore necessary to introduce mtDNA into the cells by cytoduction using a
**karl** mutant (Conde and Fink, 1976). We found that all **mpl** mutants could grow on glycerol medium and are thus capable of replicating mtDNA. Moreover, the production of cytoplasmic petites (rho-) was only slightly higher in the mutants that in the wild-type strain (Table I). However, we found that the growth on glycerol medium at 36°C was significantly reduced as compared with the wild-type strain (not shown).

Substitution of alanine or glycine for the aspartate residues D171, D230 and D347 elicited a several hundred-fold increase in the frequency of spontaneous mutations leading to erythromycin resistance (Table I). Erythromycin resistant (E^R^) mutants are invariably of mitochondrial inheritance and corresponding mutations have been mapped to two distinct alleles of the mitochondrial 21S rRNA gene. (Netter et al., 1974; Sor and Fukuhara, 1983). The **mpl** mutants have therefore a mutator activity. In contrast, replacement of the Cys344 residue by a glycine residue only increased the frequency of E^R^ mutants by 10-fold. This residue must thus contribute to ensuring faithful replication without playing a critical role. These results show that the mutated residues play an important role in mtDNA replication fidelity in vivo and strongly suggest that they are located in the 3'→5' exonucleolytic domain of the yeast mtDNA polymerase gene.

**In vitro 3'→5' exonuclease activity of the mutants**

The 3'→5' exonuclease activity was measured in the mutator strain A25[pLGALD347A (**mpl**-5)] which overproduces the mtDNA polymerase from the **GAL** promoter. It was shown by SDS–polyacrylamide gel electrophoresis that mutant and wild-type mitochondrial extracts contained the same amount of mtDNA polymerase per mg of protein (Figure 1B, panel b). Thus, the results can be readily compared.

The primer-template substrates described above were used to measure the 3'→5' exonuclease activity on mismatched 3'-hydroxyl terminus. Even after long incubation times, hardly any substrate digestion was detected (Figure 2) and it was estimated that in the mutant, the 3'→5' exonuclease activity was ~500-fold lower than in the wild-type strain.

In the three other mutator strains, the 3'→5' exonuclease

<table>
<thead>
<tr>
<th>Table II. Mitochondrial DNA polymerase activity in wild-type and mutant strains</th>
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<tbody>
<tr>
<td><strong>Gap filling</strong> (units/mg protein)</td>
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<tr>
<td>-----------------------------------</td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
</tr>
<tr>
<td>pLGALZ3</td>
</tr>
<tr>
<td>pLGALMIP1</td>
</tr>
<tr>
<td>pLGALD347A</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
</tr>
<tr>
<td>MIP1/PFL39</td>
</tr>
<tr>
<td>D347A/PFL39</td>
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</table>

Experiment 1 was carried out with the rho^+^ strain A25 grown in galactose medium and harbouring either pLGALZ3, pLGALMIP1 or pLGALD347A plasmids. Experiment 2 was carried out with the strain A25**mpl**-01 grown in complete ethanol (YE) medium and harbouring centromeric MIP1/PFL39 or D347A/PFL39 plasmids (rho^+^). Gap filling activity on activated DNA and extent of elongation on singly-primed M13 DNA were measured as described in Materials and methods.

of mtDNA polymerase activity in wild-type and mutants. The mtDNA polymerase was overproduced in the rho^+^ strain A25**mpl**-02 harbouring the **MIP1** (or **mpl**1) gene on the multicopy T7-3 plasmid (Table III). The reaction mixture contained 1.5 μM M13mpl18 DNA substrate (200–400 c.p.m./pmol). Polymerase activities in the 60 μl assays were as follows: wild-type (0.06 units); D171G (0.16 units); D230A (0.11 units); D347A (0.17 units); C344G (0.03 units).

\[\text{Fig. 4. Mismatch-specific 3'→5' exonuclease activity in wild-type strain and mutants. The mtDNA polymerase was overproduced in the rho}^+\text{ strain A25}\text{mpl}\text{1-02 harbouring the MIP1 (or mpl1) gene on the multicopy T7-3 plasmid (Table III). The reaction mixture contained 1.5 μM M13mpl18 DNA substrate (200–400 c.p.m./pmol). Polymerase activities in the 60 μl assays were as follows: wild-type (0.06 units); D171G (0.16 units); D230A (0.11 units); D347A (0.17 units); C344G (0.03 units).}\]
activity was estimated using mitochondrial extracts from strains harbouring multicopy plasmid-borne mip1 genes under the control of their own promoter. It was thus verified that in the mutant A25[D347A/T7-3] (mip1-5) as well as in the mutants D171G (mip1-2) and D230A (mip1-3), even after long incubation times (30 min) and in the presence of an excess of mtDNA polymerase units, the extent of substrate digestion was at least 100-fold lower than in the wild-type strain when standardized with the same amount of polymerase units (Figure 4). These mutant strains are thus practically devoid of 3′-5′ exonuclease activity. In the slight mutant C344G (mip1-4), the mismatched-specific 3′-5′ exonuclease activity was ~30 to 40% of that of the wild-type strain (Figure 4).

**DNA polymerase gap filling activity**

The activity of the mtDNA polymerase on DNase I-activated DNA was measured in soluble mitochondrial extracts from wild-type and D347A mutant strains (Table II). When the mtDNA polymerase was expressed from a PFL39 centromeric vector-borne MIP1 (or mip1) allele and thus was not overproduced, the specific activity of the polymerase in the mutant D347A was still 80% of that of the wild-type enzyme. When the mtDNA polymerase was expressed from the GAL1 promoter, the specific activity of wild-type enzyme was increased up to 200 to 500-fold. This activity was only slightly decreased in the mutant (Table II). It was concluded that the catalytic activity of the mutant enzyme on activated DNA is not severely affected.

The polymerase activity of the slight mutator C344G was measured in a strain harbouring the mip1 gene on the T7-3 multicopy plasmid and under the control of its own promoter. The very slight decrease of its specific activity (85% of wild-type activity) was hardly significant (not shown).

**Singly-primed M13 DNA synthesis**

As shown by alkaline agarose gel electrophoresis, singly-primed M13 DNA was efficiently elongated to the full-length product (7 kb) by wild-type mtDNA polymerase over-expressed from the GAL1 promoter (Figures 5, 6B and 6C). However, when the concentration of M13 DNA was increased, the number of fully elongated molecules reached a maximum at ~2 µM substrate while lower molecular weight intermediates started to be produced (Figure 5). These data reflect the degree of processivity of the polymerase (processivity is defined as the extent of polymerization in a single binding event) and can be explained as follows. Under conditions of substrate limitation, dissociation of the mtDNA polymerase from the substrate is followed in most instances by reassociation of the enzyme to a previously
extended 3’-hydroxyl terminus; the dissociation event is not detected because, finally, a full-length product is made. Under conditions of substrate excess (10 μM), there are more 3’-hydroxyl termini whose extension is initiated; however, after dissociation from the substrate there is a high probability (because of the excess of substrate) that the polymerase binds to 3’-hydroxyl termini which have not been extended previously, giving rise to products of intermediate size. In contrast, a perfectly processive enzyme would not dissociate from the substrate, only synthesizing full-length DNA molecules. On the contrary, a poorly processive enzyme such as E.coli Pol1 (Klenow fragment) which associates with and dissociates from the template very rapidly (Tabor et al., 1987) elongates the DNA chain at a rate that is strongly dependent on protein concentration (Figure 6D). In some respects, the yeast mtDNA polymerase resembles that of bacteriophage T7 (Tabor et al., 1987) as it finally elongates a significant number of the DNA chains under conditions of substrate excess and at a rate that is independent of protein concentration (Figure 6C). However, the mitochondrial enzyme halts DNA chain elongation at certain sites along the M13 DNA molecule. These are not randomly distributed and constitute hot spots of DNA synthesis arrest. Altogether, our data suggest that the yeast mtDNA polymerase is moderately processive; it is sensitive to template secondary structure that elicits pauses in DNA synthesis.

Because we were working with a non-purified enzyme, it cannot be excluded that processivity is enhanced by specific proteins present in the mitochondrial extracts. This implies that these processivity factors should be present in such a large excess in the mitochondrial extracts as to stoichiometrically react with mtDNA polymerase when the latter is 500-fold overexpressed from the GALI promoter. A more plausible hypothesis would be that processivity is enhanced by a non-stoichiometric factor which catalytically modifies the polymerase or interacts with the DNA substrate. However, it is also possible that yeast mtDNA polymerase itself is processive.

The extent of singly-primed M13 DNA synthesis in mitochondrial extracts of the mutant D347A was ~2-fold slower than in the wild-type strain under overproduction and non-overproduction conditions (Table II). Moreover, under assay conditions where the extent of DNA synthesis was the same in the mutant and wild-type strains, the fraction of fully elongated DNA was considerably decreased in the mutant (Figures 5, 6A, 6B and 6C). This was not due to a modifica-
tion of the $K_m$ of the mutant enzyme for M13 DNA substrate as compared with the wild-type polymerase. These observations were extended to the 3'-5' exonuclease-deficient mutants D171G and D230A, overproducing the mtDNA polymerase from their own promoter (not shown). Thus, it can be concluded that 3'-5' exonuclease deficiency elicits a dramatic decrease in the size of the products that are synthesized from singly-primed M13 DNA. This suggests that the mutant mtDNA polymerase easily dissociates from its DNA substrate because it is less processive and more sensitive to secondary structure. In contrast, the DNA products pattern of the mutant C344G was similar to that of the wild-type strain (not shown).

Discussion

This is the first report that the 3'-5' exonuclease activity associated with mitochondrial DNA polymerase plays an important role in correcting replication errors in vivo. It is in agreement with the low mutation rate of yeast mtDNA which accumulates less base substitutions than nuclear DNA (Clark-Walker, 1991). Moreover, these data obtained with yeast also strongly suggest an in vivo proofreading function for the 3'-5' exonuclease activity associated with animal mtDNA polymerases which exhibit high accuracy in vitro (Kaguni et al., 1988; Kunkel and Soni, 1988; Insdorf and Bogenhagen, 1989; Kaguni and Olson, 1989).

The yeast mitochondrial DNA polymerase overexpressed from the strong inducible GAL1 promoter was detected as a major 140 kDa polypeptide in soluble mitochondrial extracts. Under these conditions, we could show that it exhibits a potent and classical 3'-5' exonuclease activity that is more active on mispaired 3'-hydroxyl termini. In contrast with Drosophila mtDNA polymerase (Kaguni and Olson, 1989) and like the chicken and Xenopus enzymes (Kunkel and Soni, 1988; Insdorf and Bogenhagen, 1989), yeast mtDNA polymerase is able to excise correctly paired bases from a duplex. It is not clear whether in animal mtDNA polymerases the exonuclease activity belongs to the polymerase polypeptide (Kaguni and Olson, 1989; Longley and Mosbaugh, 1991). The MIPI gene is translated as a single 140 kDa polypeptide. Thus, in yeast the 3'-5' exonucleolytic domain belongs to the N-terminal part of the polymerase.

It has been found that many eukaryotic and prokaryotic DNA polymerases share three motifs (named Exo1, Exo2 and Exo3) with the 3'-5' exonuclease domain of E.coli PolI DNA polymerase (Bernad et al., 1989). We and others have found these motifs in the N-terminal part of the MIPI gene (Fourny and Vanderstraeten, 1990; Ito and Braithwaite, 1990; Blanco et al., 1991). Interestingly, MIPI consensus 3 shares with viral DNA polymerases and K.lactis killer factor DNA polymerases a cysteine residue (Tommasino et al., 1988; Bernad et al., 1989). Although these motifs have been detected in many DNA polymerases, in many cases it remains unclear whether they belong to the 3'-5' exonuclease domain. Amino acid substitutions in the consensus sequences dramatically decrease 3'-5' exonuclease activity in PolI, T7 and Φ 29 bacteriophages DNA polymerases (Joyce and Steitz, 1988; Bernad et al., 1989; Tabor and Richardson, 1989; Derbyshire et al., 1991; Patel et al., 1991) as well as in yeast DNA polymerases $\delta$ and $\epsilon$ (Morrison et al., 1991; Simon et al., 1991). This contrasts, however, with a recent report concerning bacteriophage T4 DNA polymerase (Reha-Krantz et al., 1991). In that particular report, amino acid substitutions in the presumed Exo1 site only slightly decreased the 3'-5' exonuclease activity and had only a small effect on the spontaneous mutation rate.

We have used site directed mutagenesis, replacing conserved aspartate residues with neutral alanine or glycine residues, in order to demonstrate that the three consensus sequences belong to the 3'-5' exonuclease domain of yeast mitochondrial DNA polymerase. Taking advantage of the fact that the mtDNA polymerase of the wild-type and D347A mutant strains are similarly overproduced from the GAL1 promoter, we determined that the 3'-5' exonuclease activity is 500-fold lower in the mutant than in the wild-type strain when standardized to the same number of polymerase units. A similar deficiency was observed in the other substitution mutants that exhibited a strong mutator phenotype. The ability of 3'-5' exonuclease-deficient mutants to synthesize DNA both in vivo and in vitro shows nucleotide excision to be the primary defect. Deficiency in 3'-5' exonuclease activity is reflected in vivo by a several hundred-fold higher mtDNA mutation frequency. This represents a normal contribution of proofreading to DNA replication accuracy (Fersht et al., 1982).

The effect of substituting a glycine for Cys344 residue is relatively minor; 3'-5' exonuclease activity and the mutation rate are both slightly but significantly affected. This indicates that this conserved residue is not critical to catalysis but contributes to proofreading efficiency.

We also analysed whether 3'-5' exonuclease deficiency was altering polymerization and replication activities of yeast mtDNA polymerase. When mipI alleles are harboured on a centromeric vector and transformed into cells, production of cytoplasmic petites by the 3'-5' exonuclease-deficient mutants at 30°C is only slightly increased as compared with wild-type strain. In other words, mtDNA replication and segregation into daughter cell are not severely affected in the mutants and their mutator activity is certainly mostly limited to point mutations. However, growth on glycerol at an elevated temperature is severely impaired.

In mitochondrial extracts and under conditions of substrate excess, yeast mtDNA polymerase expressed from the GAL1 promoter efficiently catalyses the elongation of singly-primed M13 DNA to full-length 7 kb product. The presence of DNA bands of intermediate size indicates, however, that the polymerase is sensitive to template secondary structure. This is also true for Drosophila mtDNA polymerase (Wernette et al., 1988). The elongation process in the mutant C344G is not altered. In 3'-5' exonuclease-deficient mutants, whether mipI alleles were harboured on centromeric or multicopy plasmids, low molecular weight DNA products accumulate preferentially to fully elongated DNA, suggesting that the mutant mtDNA polymerases fall off the expanding end of the DNA chain very easily, probably because they are highly sensitive to template secondary structure and less processive than the wild-type enzyme.

This points to tight coupling in vitro between the 3'-5' exonuclease and 5'-3' polymerisation domains of yeast mtDNA polymerase. In contrast, the polymerising activity of 3'-5' exonuclease-deficient PolI, T7 and Φ 29 polymerases is not altered (Joyce and Steitz, 1987; Bernad et al., 1989; Tabor and Richardson, 1989; Derbyshire et al., 1991; Patel et al., 1991).
This also holds for the yeast nuclear DNA polymerases δ and ε (Morrison et al., 1991; Simon et al., 1991). In PolII, X-ray crystallographic studies have revealed that exonucleolytic and polymerisation domains are separated by 30 Å and behave as distinct entities (Ollis et al., 1985). It has been shown that when the highly processive T7 DNA polymerase (Tabor et al., 1987) meets a mismatch, DNA is channelled to the exonuclease site as a consequence of delayed chain elongation at a mispaired terminus and does not dissociate from DNA polymerase in the absence of excision (Donlin et al., 1991). In contrast, mutations in the conserved regions of the 3′ → 5′ exonuclease domains of the α-like DNA polymerase from Herpes simplex virus severely impair viral DNA replication and polymerase activity (Gibbs et al., 1991), suggesting that in this polymerase, the exonuclease and polymerase domains are not well separate entities. Even though from primary structure comparisons the yeast mtDNA polymerase has been classified among type A polymerases (Blanco et al., 1991; Ito and Braithwaite, 1991), our data indicate that in contrast with these polymerases the 3′ → 5′ exonuclease domain plays a role in processivity. This is also the case for E. coli DNA polymerase III whose processivity is strictly dependent on the presence of the exonuclease subunit in the holoenzyme (Studwell and O'Donnell, 1990).

Our in vitro data have to be correlated to observations made in vivo. In vivo mtDNA replication is not severely altered. This suggests that our in vitro system imperfectly reflects in vivo physiological conditions. Accessory proteins of the replication complex or concentration of cofactors (such as magnesium) may considerably improve in vivo mtDNA replication. It must be noted, however, that when the mutants are grown at an elevated temperature (36°C), their growth on glycerol medium is severely decreased, suggesting that at this temperature in vivo replication is better reflected by our in vitro system.

Animal mitochondrial DNA polymerases are highly accurate in vitro: they select bases with high accuracy and possess a potent proofreading activity. The same accuracy applies to the yeast enzyme, as shown by the mutant phenotype of 3′ → 5′ exonuclease-deficient mutants. The several hundred-fold increase in DNA replication accuracy attributable to the mitochondrial 3′ → 5′ exonuclease editing activity is within the range of its expected contribution. Why such a high accuracy for a multicopy genomic system? Although we have no definitive answer, a justification could be the great biological importance of the mitochondrial genetic information which is essential to life in most eukaryotes and the fact that segregation mechanisms allow but a few copies of mtDNA to enter cells during meiosis. The severity and the wide variety of mitochondrial diseases (Wallace, 1989; Lander and Lodish, 1990) emphasize the reliance of mitochondria on error-free replication systems.

### Materials and methods

#### Strains and media

Yeast strains and plasmids are listed in Table III. Strains were grown on YD (2% glucose, 2% yeast extract Kat), YG (2% glycerol, 2% yeast extract), YE (2.5% ethanol, 2% yeast extract) or minimal medium (either 2% glucose, galactose or raffinose plus 0.1% glucose; 0.7% yeast nitrogen base) supplemented with required amino acids and bases. Solid media were supplemented with 2% agar. When indicated, YG medium was supplemented with 50 mM sodium phosphate, pH 6.5 and 4 mg/ml erythromycin.

### Table III. List of yeast strains and plasmids

<table>
<thead>
<tr>
<th>Strains or plasmids</th>
<th>Relevant characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saccharomyces cerevisiae</td>
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</tr>
<tr>
<td>W303-1B</td>
<td>Matta ade2 ura3 his3 trp1 leu2 rho+</td>
</tr>
<tr>
<td>W303-1B/LΔmipl-01</td>
<td>as above, but LEU2::URA3 rho0</td>
</tr>
<tr>
<td>A25</td>
<td>Matta ade2 ura3 trp1::LEU2 rho+</td>
</tr>
<tr>
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<td>as above, but mipl::URA3 rho0</td>
</tr>
<tr>
<td>A25Δmipl-02</td>
<td>as above, but mipl::TRP1 rho0</td>
</tr>
<tr>
<td>JC7</td>
<td>Mata leu1 kar1 rho+</td>
</tr>
<tr>
<td>Centromeric plasmids</td>
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<tr>
<td>PFL39</td>
<td>PUC19 vector with a BglII cassette containing the TRP1 gene and a C Pacers cassette containing CENVI plus ARS (from F.Lacroute)</td>
</tr>
<tr>
<td>MIP1/PFL39</td>
<td>MIP1 insert in Smal site of PFL39 plinker</td>
</tr>
<tr>
<td>D171G/PFL39</td>
<td>as above, but mipl-2</td>
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<tr>
<td>D230A/PFL39</td>
<td>as above, but mipl-3</td>
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<td>C344G/PFL39</td>
<td>as above, but mipl-4</td>
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<td>D347A/PFL39</td>
<td>as above, but mipl-5</td>
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<td>2 μ episomal plasmids</td>
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<td>YEp24</td>
<td>Yeast multicopy vector</td>
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<td>T7-3</td>
<td>Selected from a library constructed in YEp24 plasmid (Foury, 1989); contains a 6 kb insert including the MIP1 and VMA4 genes</td>
</tr>
<tr>
<td>D171G/T7-3</td>
<td>as above, but mipl-2</td>
</tr>
<tr>
<td>D230A/T7-3</td>
<td>as above, but mipl-3</td>
</tr>
<tr>
<td>C344G/T7-3</td>
<td>as above, but mipl-4</td>
</tr>
<tr>
<td>D347A/T7-3</td>
<td>as above, but mipl-5</td>
</tr>
<tr>
<td>plGALZ3</td>
<td>Yeast multicopy vector containing the GAlI promoter (Lahaye et al., 1991)</td>
</tr>
<tr>
<td>plGALMP1</td>
<td>pLGALZ3 after deletion of lacZ gene and insertion of MIP1 gene</td>
</tr>
<tr>
<td>plGALD347A</td>
<td>as above, but mipl-5</td>
</tr>
</tbody>
</table>

The construction of the disrupted mipl::URA3 gene has been reported by Foury (1989). The disruption mipl::TRP1 was performed by inserting the TRP1 gene contained in a BglII cassette into the unique BglII site of the MIP1 gene. The wild-type LEU2 marker was introduced into the strain W303-1B/LΔmipl-01 by integrative transformation, giving rise to the strain W303-1B/LΔmipl-01. The strain A25 has been isolated as previously reported (Lahaye et al., 1991) and contains a disrupted copy of the nuc1 gene (Zassenhaus et al., 1988) constructed by P Zassenhaus (University of Saint Louis). In this study, all strains harbouring plasmids are rho+. The vector PFL39 is a gift from F Lacroute (Centre de Génétique Moléculaire, Gif-sur-Yvette, France). The vector YEp24 has been described by Botstein et al. (1979). The VMA4 gene has been described by Foury (1990). The vector pLGALZ3 has been described by Lahaye et al. (1991).

### Nomenclature

The MIP1 and mipl were borne on either multicopy or centromeric plasmids. In all experiments, except those utilizing the GALI promoter, the plasmids were present in a yeast strain that had a deleted copy of the chromosomal MIP1 gene (Δmipl-01 or Δmipl-02, see Table III). However, the transformation experiments using multicopy plasmids with the MIP1 (or mipl-5) gene fused to the GALI promoter (pLGALMP1 or pLGALD347A plasmids) were carried out in the rho+ strain A25 which still possesses the chromosomal copy of MIP1. Thus after transformation with the pLGALD347A plasmid, a single MIP1 copy and a large number of mipl-5 copies are simultaneously present in the cells. Although heterozygous for the MIP1 gene, these transfomants were also designated as 'mutants' in our text for the following reason. The mutant polypeptide expressed from the GALI promoter in the plasmid pLGALD347A is in such large amounts compared with the protein encoded by the chromosomal wild-type MIP1 that the latter is not detected (or hardly detected) in the enzymatic assays. Strains whose MIP1 (or mipl) gene is harboured on a centromeric vector...
Site directed mutagenesis and construction of mutants

Standard oligonucleotide mutagenesis procedures were used to introduce point mutations in the M1 gene (Sambrook et al., 1989; Stanisens et al., 1989). The M1 nucleotide sequence is numbered according to Fourny (1989). The restriction fragments subjected to site directed mutagenesis were entirely sequenced by the dideoxynucleotide chain termination method (Biggins et al., 1983). Thus, it was verified that they did not contain additional nucleotide alterations. The mutated restriction fragments were substituted for their wild-type homologues in the centromeric M1/PFL39 plasmid or in the multicopy T7-3 plasmid. The disrupted mipl strain W303-IB/L-Δmipl-01 and A25Δmipl-02 were transformed with the centromeric plasmids and the disrupted mipl strain A25Δmipl-02 was transformed with the multicopy plasmids. In order to introduce mtDNA into the cells transformants were crossed on YD medium with the rho+ strain JC7 which bears the karl mutation and the mixture of haploids, diploids and cytoductants was streaked onto minimal glucose medium devoid of leucine to eliminate JC7 haploids and select mutant cytoductants.

The plasmids that contain M1 under the control of the inducible GAL promoter were constructed as follows. A SalI site created by site direct mutagenesis (G to C change at position 515), 45 bp upstream of the initiator ATG codon (Figure 1A). A 4.2 kb SalI–PvuII fragment containing the M1 gene and 3′ flanking sequences was substituted for the SalI–PvuII fragment of the pLGALZ3 vector which contains most of the lacZ gene. The D347A mutant plasmid was constructed by substituting the mutated BglII–PvuII fragment (characterized by a new BglII site) for its wild-type homologue.

Mitochondrial DNA mutations frequency

The strain W303-IB/L-Δmipl-01/rho− containing M1/PFL39 or mipl/PFL39 plasmids was used to estimate the frequency of spontaneous mtDNA mutations. 10–15 independent cultures for the wild-type strain were inoculated from independent colonies and grown in liquid complete glycerol (YG) medium for 2 days at 30°C. Aliquots were spread in lawns on glycerol plates (YG) supplemented with 4 mg/ml erythromycin. The number of erythromycin resistant (E8) colonies was determined after 5 days at 30°C. This experiment was repeated several times. The mutant activity was estimated as the average number of E8 colonies per 106 cells in the culture. The frequency of rho− mutants estimated as follows. Cells were grown for 24 h in liquid ethanol (YE) medium and spread on YE plates for single colonies. The percentage of rho− colonies on YE plates was determined from the size of the colonies. The rho− colonies which had lost their plasmid were subtracted.

Mitochondrial DNA polymerase extract

The different conditions of culture were as follows. The wild-type A25Δmipl-01/M1/PFL39 and the corresponding mutant D347A were grown in ethanol medium (YE) until late exponential phase of growth. The wild-type strain A25Δmipl-02/M1/T7-3 rho− and the mutant strains A25Δmipl-02/M1/T7-3 rho− were grown in minimal raffinose medium until late exponential phase. The A25 strains with the multicopy plasmidborne M1 (or mipl) gene under the control of the GAL1 promoter were first grown in raffinose minimal medium (without glucose) for 24 h. This culture was used to inoculate the galactose minimal medium at a final density of ~5 × 106 cells/ml and galactose induction was proceeded for 16 h. Soluble mitochondrial extracts enriched in mtDNA polymerase were prepared as previously reported (Fourny, 1989), except that protease inhibitors (1 µg/ml leupeptine and aprotinin, 2 mM benzamidine and 1 mM PMSF) were added during protoplasting and mtDNA polymerase solubilisation steps. PMSF (1 mM) was also added during protoplasting in galactose-grown cells.

Gap filling activity of mitochondrial polymerases

The DNA polymerase activity was measured at 37°C in a 200 µl reaction mixture containing 20 mM Tris–HCl, pH 8.0, 50 mM MgCl2, 25 µM each of dATP, dCTP, dGTP and 5.5 µM [3H]dUTP (3000 c.p.m./pmol), 2 mM dihydrothioctic acid and 200 µM DNAse-activated calf thymus DNA. Aliquots of 40 µl were removed at time intervals and the radioactivity was measured as previously reported (Genga et al., 1986). The unit of mtDNA polymerase activity is defined as the amount of enzyme which catalyses the incorporation of 1 nmol dNTP into acid-insoluble material in 60 min at 37°C with activated calf thymus DNA as the substrate.

3′–5′ exonuclease activity on mismatched 3′-hydroxyl terminus

A 17 mer oligonucleotide that was either perfectly complementary to the M13 sequence (AB353, GTAAAAACGGCCCGAAT) or contained a single mismatch dAMP: dAMP at the 3′ hydroxyl terminus (AB354, GTAAAACGGACGCCGAAT) was annealed to M13mp19 (or mp18) ssDNA. Oligonucleotides were previously labelled at their 5′ end with [γ-32P]dATP and T4 polymerase kinase. An excess of primer was annealed to 5 µM M13 DNA in a 50 µl hybridization buffer (10 mM Tris–HCl, pH 7.5, 100 mM KCl) at 70°C for 5 min followed by cooling to room temperature. Singly-primed DNA was purified on a Sepharose CL-4B column in a Pasteur pipette equilibrated with 80 mM NaCl and 10 mM Tris–HCl, pH 7.5. The exonuclease reaction mixture (50–60 µl) contained 20 mM Tris–HCl, pH 8.0, 50 mM MgCl2, 0.5 mg/ml bovine serum albumin (BSA), 3 mM dithiothreitol (DTT) and 2.4 µM DNA substrate (~200 c.p.m./pmol). The reaction was started with the mitochondrial DNA polymerase extract and carried out at 30°C. Aliquots of 8 µl were removed at identical times and quenched with an equal volume of 80% formamide, 0.75% sodium dodecylsulfate (SDS) and 3 mM EDTA, pH 8.0. Products were analysed by electrophoresis in 15% polyacrylamide–8 M urea gels and autoradiography of the gels. The extent of 17mer hydrolysis was quantified by cutting the bands from the autoradiography into small pieces followed by elution of the silver grains with 1 N NaOH and measurement of the optical density at 500 nm in the colloidal medium (Sussia, 1983). Blanks were carried out by cutting pieces of the film that have not been exposed to the radioactivity.

Singly-primed M13 DNA synthesis

Annealing with AB353 primer and singly-primed DNA purification were performed as described above. The experiments were carried out either with DNA that was [32P]labelled at the 5′ end of the oligonucleotide as reported above or with unlabelled DNA which was elongated in the presence of [α-32P]dCTP. In the latter case, the DNA elongation reaction mixture (90 µl) contained 20 mM Tris–HCl, pH 8.0, 5 mM DTT, 0.5 mg/ml BSA, 25 mM KCl, 35 mM MgCl2, 25 µM dATP, dCTP, dGTP, 5 µM [α-32P]dCTP (500 to 20,000 c.p.m./pmol) and singly-primed M13 DNA at the indicated concentration. In the former case, dCTP was unlabelled and its concentration was 25 µM. The reaction was started with the mitochondrial DNA polymerase extracts and carried out at 37°C. Aliquots of 20 µl were removed at indicated times and incubated further at 37°C for 20 min with 5 µl of stopper buffer containing 1 mg/ml proteinase K, 5% sodium dodecylsulfate (SDS) and 0.5 M NaCl, pH 8.0. Before electrophoresis in a 1% agarose gel (Sambrook et al., 1989), DNA was phenol-extracted and denatured for 15 min at room temperature in a buffer containing 30 mM NaOH, 1 mM EDTA and 50% glycerol. Gels were fixed, dried and autoradiographed. Quantification of the extent of DNA synthesis was carried out in parallel experiments under the same reaction conditions, except that 5 µM [α-32P]dCTP were used. Aliquots were removed at indicated times, spotted onto Whatman GF/C filters, precipitated into 5% trichloroacetic acid and washed as previously reported (Genga et al., 1986).

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References


Mutators of yeast mitochondrial DNA polymerase


